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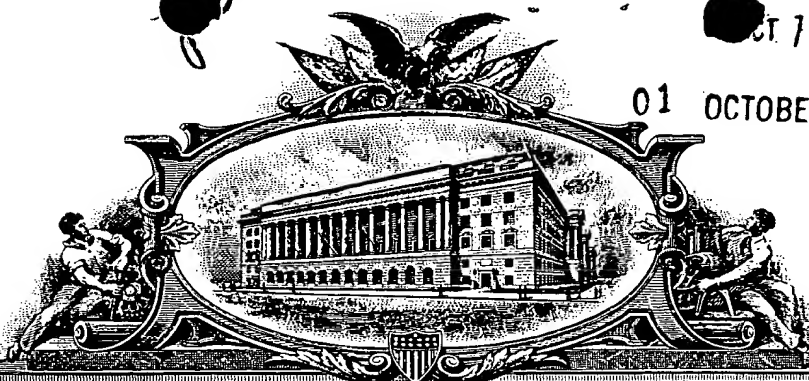
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THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 08/576,238

FILING DATE: December 21, 1995

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

T. Lawrence

T. LAWRENCE
Certifying Officer



A/10/07/EC38

**A STABILIZED THIN FILM OF CALCIUM PHOSPHATE ENTITIES
PARTICULARLY ADAPTED FOR BONE CELL CULTURE**

FIELD OF THE INVENTION

5 This invention relates to thin films of calcium phosphate entities which are capable of supporting bone cell activity thereon. The thin film of calcium phosphate entities is stabilized by the presence of silicon entities in conjunction with hydroxyapatite and α -tricalcium phosphate.

BACKGROUND OF THE INVENTION

10 Bone is a complex mineralizing system composed of an inorganic or mineral phase, an organic matrix phase, and water. The inorganic mineral phase is composed of crystalline calcium phosphate salts while the organic matrix phase consists mostly of collagen and other noncollagenous proteins. Calcification of bone depends on the close association between the organic and
15 inorganic phases to produce a mineralized tissue.

 The process of bone growth is regulated to meet both structural and mechanical stresses. The cells involved in the processes of bone formation, maintenance, and resorption are osteoblasts, osteocytes, and osteoclasts. Osteoblasts synthesize the organic matrix, osteoid, of bone which after calcium
20 phosphate crystal growth and collagen assembly becomes mineralized. Osteocytes regulate the flux of calcium and phosphate between the bone mineral and the extracellular fluid. Osteoclasts function to resorb bone and are essential in the continuous process of bone remodelling. Disturbing the natural balance of bone formation and resorption leads to various bone disorders.
25 Increased osteoclast activity has been demonstrated to lead to bone disease characterized by a decrease in bone density such as that seen in osteoporosis, osteitis fibrosa and in Paget's disease. All of these diseases are a result of increased bone resorption.

 In order to understand the mechanisms involved which regulate bone
30 cell functioning, it is important to be able to assess the normal function of bone

cells and also the degree of perturbation of this activity in various bone diseases. This will lead to the identification of drugs targeted to restore abnormal bone cell activity back to within normal levels.

Several research groups have developed methods to directly observe the activity of isolated osteoclasts *in vitro*. Osteoclasts, isolated from bone marrow cell populations, have been cultured on thin slices of natural materials such as sperm whale dentine (Boyde et al Brit. Dent. J. 156, 216, 1984) or bone (Chambers et al J. Cell Sci. 66, 383, 1984). The latter group have been able to show that this resorptive activity is not possessed by other cells of the mononuclear phagocyte series (Chambers & Horton, Calcif Tissue Int. 36, 556, 1984). More recent attempts to use other cell culture techniques to study osteoclast lineage have still had to rely on the use of cortical bone slices (Amano et al. and Kerby et al J. Bone & Min. Res. 7(3)) for which the quantitation of resorptive activity relies upon either two dimensional analysis of resorption pit areas of variable depth or stereo mapping of the resorption volume. Such techniques provide at best an accuracy of approximately 50% when assessing resorption of relatively thick substrata. In addition these analysis techniques are also very time consuming and require highly specialized equipment and training. Furthermore, the preparation and subsequent examination of bone or dentine slices is neither an easy nor practical method for the assessment of osteoclast activity.

The use of artificial calcium phosphate preparations as substrata for osteoclast cultures has also met with little success. Jones et al (Anat. Embryol 170, 247, 1984) reported that osteoclasts resorb synthetic apatites *in vitro* but failed to provide experimental evidence to support this observation. Shimizu et al (Bone and Mineral 6, 261, 1989) have reported that isolated osteoclasts resorb only devitalized bone surfaces and not synthetic calcium hydroxyapatite. These results would indicate that functional osteoclasts are difficult to culture *in vitro*.

Applicant's published international PCT patent application WO94/26872 describes a sintering process for forming thin films of calcium phosphate entities on which bone cell function occurs. This is believed to be the first thin layer of synthetic material on which osteoclasts can exhibit extended activity.

5 As described in that application, a variety of factors should be considered in providing in the thin film the desired ratio of hydroxyapatite to tricalcium phosphate. Such parameters include:

- 1) amounts of reagents for preparing the sol-gel of hydroxyapatite;
- 2) rate of combination of reagents;
- 10 3) duration and rate of mixing when making the sol-gel;
- 4) rates and methods of precipitation and separation;
- 5) process environmental conditions during the manufacture of the sol-gel;
- 6) velocity of removal of the substrate from the sol-gel in dip
15 coating a film thereon;
- 7) sintering temperature;
- 8) sintering in a controlled atmosphere such inert gas, a vacuum or
an atmosphere with water vapour present.

It was therefore suggested in the earlier PCT patent application, that in
20 order to obtain a broad range in ratios of hydroxyapatite to tricalcium phosphate, many of these parameters need to be considered in order to achieve the ratios of 10:90 through to 90:10. The suggested sintering temperatures in an air atmosphere were from approximately 800°C to approximately 1100°. It was established that at 800°C the film was predominantly hydroxyapatite, that
25 is a ratio of approximately 90:10. A sintering temperature of about 900°C provided ratios of about 70:30. At 1000°C, the ratio was about 10:90 and at 1100°C the film was predominantly tricalcium phosphate. It was also suggested that sintering in a vacuum at a 1000°C produced a ratio of approximately 66:34. It has now been found that the preferred ratios are from
30 50:50 to 20:80. The optimum ratio is approximately 333:666. To achieve these

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ratios, consideration can be given to several of the above factors. However, it is desirable to minimize the variability in several of the above factors and achieve the desired ratios for optimum film compositions in an exacting reproducible manner.

5 Applicants have found that the formation of the sol-gel, in accordance with the standard technique described in their earlier published PCT application WO94/26872 in combination with selected sintering temperatures as described in co-pending U.S. application Sr. No. _____ filed _____ and
10 entitled "Improved Sintering Process for Producing Thin Films of Calcium Phosphate Entities", achieves selected desired ratios for hydroxyapatite and α -tricalcium phosphate in the sintering film. Surprisingly, this film is stable in the presence of various aqueous media, even though α -tricalcium phosphate is supposed to be soluble in water. Applicants now discover the presence of
15 silicon entities which are believed to stabilize the film and prevent its degradation in aqueous medium. Hence, disappearance of calcium phosphate entities from the film is due to the activity of the osteoclasts and not due to a dissolution process.

SUMMARY OF THE INVENTION

20 In accordance with an aspect of the invention, a stabilized composition of calcium phosphate entities for supporting bone cell activity is provided as a sintered thin layer on a face of a substrate of quartz. The composition comprises a gradient layer of predominantly hydroxyapatite adjacent the quartz, increasing amounts of α -tricalcium phosphate at the layer surface with silicon
25 entities diffused throughout the gradient of calcium phosphate entities. The silicon entities are released from the quartz and diffused through the layer during sintering of a hydroxyapatite composition applied as a film to the face of said quartz substrate. The composition comprises a ratio of hydroxyapatite to α -tricalcium phosphate in the range of 50:50 to 20:80.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a magnified micrograph of the thin layer in accordance with an embodiment of this invention on the face of a quartz substrate; and

Figure 2 comprises graphs (a), (b) and (c) which show results of energy dispersive x-ray spectroscopy (a) at the interface of the composition with the substrate; (b) just above the interface and (c) at the top of the film.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The preferred film composition of calcium phosphate entities is that provided in accordance with the improved sintering process described in applicant's co-pending U.S. application Sr. No. _____ filed _____ and entitled "Improved Sintering Process for Producing Thin Films of Calcium Phosphate Entities". This process provides on a consistent basis, a thin film of calcium phosphate entities which are within the desired range of 50:50 to 20:80 for the ratio of hydroxyapatite to α -tricalcium phosphate. It has now been found that by virtue of providing a diffusion of silicon entities through the thin layer during the sintering process, significant unexpected stability of the α -tricalcium phosphate entity within the thin layer is provided. In accordance with a preferred aspect of this invention, the source of silicon entities can be provided by sintering a film of hydroxyapatite on the face of a quartz substrate. During the sintering period, silicon entities are released from the surface of the quartz and diffuse towards the surface of the thin layer during the conversion of the hydroxyapatite into α -tricalcium phosphate within the preferred range for the ratios of hydroxyapatite to α -tricalcium phosphate. Although it is not readily understood what role the silicon entities play in stabilizing the thin layer and preventing dissolution of the α -tricalcium phosphate, it is believed that the silicon entities form a stable complex with the α -tricalcium phosphate as it is formed by the conversion of the hydroxyapatite into α -tricalcium phosphate.

We have managed to demonstrate on a consistent basis, that the silicon entities diffuse through a gradient layer of the hydroxyapatite and the formed α -

tricalcium phosphate. Absence of such silicon ion diffusion during the sintering process, does not appear to provide a composition which exhibits stability and/or cell surface friendliness in order to achieve the desired bone cell activity.

5 The thin film as provided on a suitable support, in accordance with this invention, significantly advances the study and understanding of bone cell functional properties. The make-up of the film, as provided in accordance with this invention, permits the culture of various types of bone cells thereon. The surface make-up may be adjusted to encourage a significant degree of
10 resorption of the calcium phosphate entities of the film material through to a negligible degree of resorption of the calcium phosphate entities in the study of osteoclast activity. Similarly, osteoblast activity may be studied by detecting a build up of bone-like material. The ability to provide the material in a film which is sufficiently thin that resorption of the entities by osteoclasts can be
15 detected by the disappearance of resorbed calcium phosphate entities provides a simple inexpensive format for analysis compared to the prior art techniques. The film make-up as made in accordance with this invention, supports the biological function of bone cells. The benefit in providing the film on a transparent supporting substrate, such as quartz or glass, lends to easy
20 evaluation techniques of the diagnostic process.

 Ideally the film thickness is greater than 0.1 micron because it has been found that at film thicknesses less than 0.1 microns it is difficult to obtain uniform film coverage, free from discrete voids. As to the upper thickness limit for the film, it can be of any desired thickness depending upon its end use. As
25 will be discussed, the degree of resorption may be detected by light transmittance, which preferably requires a film less than 10 microns in thickness. The substrate is of quartz which readily withstands these temperatures and has the desired degree of transparency to permit light transmittance tests to determine the extent of resorption of calcium phosphate
30 entities from the film material.

The developen thin films may be used in kits and the like to provide for assessment of bone cell activity. The film may be embodied in the form of a "kit" comprising quartz substrates, pre-coated with an adherent calcium phosphate thin film, which may be used in a cell culture vessel (possibly a 24-well optionally sterilized multi-well plate i.e. of approximately 15 mm diameter) as a system suitable for the culture of mixed bone cell populations. The device is simple and relies on only routine laboratory equipment and techniques for use, is suitable for quantitative analysis, and is inexpensive to fabricate but strong enough to withstand normal levels of handling and may be packaged in lots, of (for example) 24 samples in a plastic presentation box. The thin film surfaces have a defined and reproducible chemistry and are mechanically strong enough to withstand transport when used with an appropriate packing material.

Modifications of the device could be designed for specific applications. For example, each substrate could be presented in a plastic support ring. The latter could be employed not only as a packaging spacer, and thus be sterilized with the substrate, but also a lip to prevent spread of culture medium and cells from the substrate itself and thus facilitate quantification of the resorptive activity. Such protection rings could also be used as stacking devices to enable multiple substrates to be employed simultaneously in the same culture well. The latter could then be enclosed in a sealed culture vessel supplied with circulating medium and could also be adopted for low and zero gravitational environments.

In each case the culture conditions may be such that osteoclasts, in either mononuclear or multinucleate form could be expected to survive in a functional state and resorb the artificial calcium phosphate of the film.

These substrates may be used to assess the resorptive activity of osteoclasts and monitor the change in this level of resorptive activity either as a result of a disease process or the inclusion, in the culture medium, of an agent

such as a drug which would influence, either directly or indirectly, osteoclastic resorptive activity.

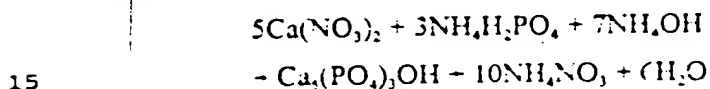
The device may be used as a means of quantifying the resorptive activity of osteoclasts or build-up of bone-like material by the activity of osteoblasts.

5 Such activity analysis may occur under continuous real-time monitoring, time-lapse intervals or end-point determination. The steps in establishing osteoclast activity are common to each of the above monitoring schedules in that bone cells (either animal or human) are cultured, in specific conditions, on one or more of the devices. The culture period is from several hours to many days and
10 preferably from approximately 2 to 10 days (the optimum time is cell species and protocol dependent), during which time the extent of osteoclast activity may be continuously monitored, periodically monitored, or simply not monitored on an on-going basis in favour of final-end-point determination. Similarly, osteoblast activity may be observed by determining extent of bone-
15 like material build-up.

Although the provision of pure or essentially pure hydroxyapatite was understood to be the calcium phosphate entity of choice in making the film, we have determined that films which are predominantly of hydroxyapatite do not encourage normal function of osteoclasts and osteoblasts and, in actual fact, in
20 the presence of osteoclasts, very little activity can be observed. It has been found, however, that by providing a mixture of calcium phosphate entities which include hydroxyapatite and α -tricalcium phosphate, the degree of resorption is encouraged through a broad range where the film predominantly of α -tricalcium phosphate provides the highest degree of resorption, whereas a
25 film predominantly of hydroxyapatite provides a negligible degree of resorption. It is this realization, with respect to the presence of α -tricalcium phosphate that explains the failure of other calcium phosphate films to encourage functional properties in osteoclasts being cultured on the films. This aspect, in providing a thin film which permits, for example, transmittance of

light or light reflection, allows one to carry out diagnostic procedures to evaluate several functional properties of bone cells being cultured on the films.

Surprisingly, it has been found that standardizing the preparation of the sol-gel and selecting a very specific range of sintering temperatures, not only achieves the desired ratios but also reveals that the optimum composition is formed by conversion of hydroxyapatite as prepared by the sol-gel process to α -tricalcium phosphate. Little or no β -tricalcium phosphates have been detected in these preferred optimized film compositions. There is no need to prepare a sol-gel which is a combination of hydroxyapatite and α -tricalcium phosphate. Instead, the technique as described in published PCT application WO 94/26872 is sufficient in preparing a sol-gel of hydroxyapatite. The chemical reaction for making hydroxyapatite in a medium of elevated pH is as follows:



The hydroxyapatite is stable in neutral and/or alkaline media. Preferably the reaction medium is brought to an elevated pH usually in the range of about 12. The phosphate solution is added drop by drop into the calcium solution to prevent the formation of tetracalcium monohydrogen triphosphate thereby the desired hydroxyapatite.

Once the sol-gel is prepared, it may be applied as a thin film to the quartz substrate in a variety of techniques. For example, the dip-coating method (C.J. Brinker et al., Fundamentals of Sol-Gel Dip Coating, Thin Solid Films, Vol. 201, No. 1, 97-108, 1991) consists of a series of processes: withdrawal of the substrate from a sol or solution at a constant speed, drying the coated liquid film at a suitable temperature, and firing the film to a final ceramic.

In spin-coating the sol-gel is dropped on a plate which is rotating at a speed sufficient to distribute the solution uniformly by centrifugal action. Subsequent treatments are the same as those of dip coating.

It is appreciated that there are a variety of other techniques which may be used to apply a thin film of the sol-gel to the substrate. Other techniques include a spraying of the sol gel, roller application of the sol-gel, spreading of the sol-gel and painting of the sol-gel.

5 An alternative to coating discrete discs of a singular size is to coat an enlarged substrate with a film of the sol-gel. The entire film on the substrate is then sintered. A device, such as a grid, may then be applied over the film to divide it into a plurality of discrete test zones. An improvement on this arrangement is described in applicant's co-pending U.S. application entitled
10 "Multi-Well Bone Cell Culture Device for Use in Assessment of Bone Cell Activity", Serial No. _____, filed _____.

In these various techniques of the sol-gel application, the thickness and quality (porosity, microstructure, crystalline state and uniformity) of formed films are affected by many factors. These include the physical properties,
15 composition and concentration of the starting sol, the cleanliness of the substrate surface, withdrawal speed of the substrate and the firing temperature. In general the thickness depends mainly on the withdrawal rate and sol viscosity for a dip coating process. Since heterogeneity in the sol is responsible for the formation of macropores and cracks, the coating operation should be
20 undertaken in a clean room to avoid particulate contamination of the sol. At the heat-treatment stage, high temperatures are required to develop the required microstructure and desired conversion of hydroxyapatite to α -tricalcium phosphate.

The purpose of applying the dip coating method to fabricate calcium
25 phosphate films is twofold: (a) to make films with required qualities (uniformity, thickness, porosity, etc.); and (b) to make translucent calcium phosphate films on transparent substrates for biological experiments.

It has been surprisingly found that sintering of the dried film of hydroxyapatite may be carried out in a standard type of high temperature oven
30 without any need to control the atmosphere in the oven. When a new oven is

used or an oven contaminated by previous use for other purposes, it is preferred to cycle the oven through the sintering temperature range several times while the oven is empty. Such pre-conditioning of the oven removes any volatiles and prepares it for use. No additional steps are required. Ambient air may be present in the oven during the break-in period and during normal use for sintering coated substrates where the presence of ambient air does not hamper the process and results in producing consistent results for the desired ratio. Under these conditions, the sintering temperature may range from 920°C up to 1100°C in providing the desired ratios of 50:50 up to 20:80. It has been found that as the temperature increases, the conversion of hydroxyapatite into α -tricalcium phosphate is also increased. At sintering temperatures in the range of 920°C up to 950°C the ratio may vary from 50:50 towards 333:666. At selected sintering temperatures in the range of 950°C to 1000° the ratio is approximately 333:666. Increasing the temperature beyond a 1000°C and up to 1100°C further increases the conversion and produces compositions having ratios in the range of 333:666 to 20:80.

The preferred sintering temperature is approximately 975°C where the ratio of 333:666 is achieved.

With reference to Figure 1, a cross-sectional TEM (defined) micrograph of the thin film shows a gradient of layers in the form of the quartz substrate (a), the interface layer (b), which is predominantly of hydroxyapatite and the upper layer (c) which includes the surface of the film and which is predominantly α -tricalcium phosphate. As it is appreciated, this micrograph is considerably enlarged as indicated by the scale of Figure 1 of 100 nm. During the sintering process, silicon entities are released from the quartz (a) and diffuse through the hydroxyapatite as it is converted to a α -tricalcium phosphate in forming the sintered thin layer. The gradient of the calcium phosphate entities is apparent from the variation in the density of the thin film cross-section where the thin film gradually progresses to a convoluted microporous structure towards the film surface. Specifically, the interface layer (b) of predominantly

hydroxyapatite appears as small rounded masses which have a granular texture. This layer (b) integrates via a translucent yet granular looking matrix with the surface (c) of the film of predominantly α -tricalcium phosphate which appears as tooth-like protrusions having a spongy or vacuolar texture, where such texture is surface friendly to bone cells to thereby promote their activity.

With reference to Figure 2, the energy dispersive x-ray spectroscopy of the substrate indicates a very high level of silicon entities with very low, if any, levels of calcium phosphate entities. the peak marked "P" for phosphate is very low, the two calcium peaks indicate respectively the higher peak hydroxyapatite, whereas the lower peak, the presence of α -tricalcium phosphate. The x-ray spectroscopy for the interface at (b) indicates the presence of silicon entities along with a very high level of hydroxyapatite as indicated by the first calcium peak and increased levels of α -tricalcium phosphate as indicated by the second calcium peak. The diffusion of silicon entities provides the greatest of concentration in the interface layer. However, as shown in Figure 2 (c), the silicon entities diffuse to the surface for the upper layer (c) of Figure 1, where the level of hydroxyapatite has decreased relative to the amount of α -tricalcium phosphate indicated by the second calcium peak. It is this presence of the silicon entities in the interface as well as the top layer which stabilizes the α -tricalcium phosphate to provide a cell friendly surface on which bone cell activity can readily occur and where the α -tricalcium phosphate is not simply removed from the surface by dissolution which had been a problem in the past with α -tricalcium phosphate layers.

The following procedures exemplify aspects of the invention for providing a thin layer composition which has the diffused silicon entities therein.

PROCEDURE 1

The following procedure is based on preparing sufficient sol-gel to coat a limited number of substrate discs. As per the above-noted chemical reaction,

Solution A comprises a calcium nitrate which is preferably calcium nitrate tetrahydrate. Solution B comprises an ammonium phosphate which is preferably ammonium dihydrogen orthophosphate (mono basic). Solution A is mixed with Solution B to produce the desired sol-gel, Solution C. Solution A is prepared by adding 40 mls of doubly distilled water to 4.722 grams of calcium nitrate - $\text{Ca}(\text{NO}_3)_2$. The solution is stirred at moderate speed for sufficient time to dissolve all of the calcium nitrate which is normally in the range of 3 minutes. To this solution, 3 mls of ammonia hydroxide (NH_4OH) is added and stirred for approximately another 3 minutes. The pH of the solution is tested where a pH of about 12 is desired. To this solution is added 37 mls of double distilled water to provide a total solution volume of approximately 80 mls. The solution is stirred for another 7 minutes and covered.

Solution B is prepared by adding 60 mls of double distilled water to a beaker containing 1.382 grams of $\text{NH}_4\text{H}_2\text{PO}_4$. The beaker is covered and stirred at moderate speed for 3 to 4 minutes until all $\text{NH}_4\text{H}_2\text{PO}_4$ is dissolved. To this solution is added 71 mls of NH_4OH and the beaker then covered and stirring continued for approximately another 7 minutes. The pH of the solution is tested where a pH of about 12 is desired. To this is added another 61 mls of double distilled water and the beaker covered to provide a total solution volume of approximately 192 mls. The solution is then stirred for a further 7 minutes and covered.

The desired sol-gel is then prepared by combining Solution B with Solution A. All of Solution A is introduced to a 500 ml reagent bottle. Stirring is commenced at a moderate speed and Solution B introduced to the reagent bottle at a rate of approximately 256 mls per hour until all 192 ml of Solution B is delivered into Solution A. An excess of Solution B may be used to compensate for any solution which may remain in the 250 ml beaker or any tubing used in the transfer process. After completion of this addition and combination of Solution A with Solution B, the resultant Solution is continued to be stirred at moderate speed for approximately 23 to 24 hours. The resultant

sol-gel is inspected for any abnormal precipitation or agglomeration. If any abnormal precipitation or agglomeration has occurred, the solution must be discarded and preparation commenced again. The sol is then carefully transferred to another 500 ml reagent bottle so as to avoid any inclusion of particle agglomerations that may be present on the walls of the original reagent bottle. Approximately 240 mls of Solution C, that is the resultant sol, is delivered to a centrifuge bottle and centrifuged for 20 minutes at about 500 rpm at room temperature. Following centrifugation, 180 mls of supernatant is discarded without disturbing the sediments. The sediments are gently resuspended by mixing in a smooth rotating manner for about 30 minutes. Viscosity of the sol is then measured and preferably is between 20 to 60 cP. The sol is then ready for dip coating of the selected substrate.

PROCEDURE 2

Cleaning of quartz discs is achieved by placing the discs in a glass beaker and supplying chromic acid cleaning solution to the glass beaker to cover all discs. The beaker is then covered. The discs are then sonicated in a water bath for 1 hour. The acid is washed away using tap water for 20 minutes. The residual tap water is removed by three changes of doubly distilled water. After the final change of double distilled water, every single disc is dried with lint-free towel and inspected for flaws in the quartz surface. Any residual particulate on the surface is removed as needed with compressed nitrogen or air. The discs are stored in covered trays in an aseptic environment.

PROCEDURE 3

The quartz disc substrate is dipped in the sol prepared by Procedure 1. The disc is grasped at the edges to avoid touching the surface. The disc is dipped in the sol, preferably by machine. The disc is removed from the sol at a prescribed withdrawing velocity. The coating on one side of the disc is removed. The coated substrate is then placed in a clean petri dish and covered

and dried at room temperature. The film, as formed prior to sintering, should be uniform without cracks, clumps or voids. It is understood that the dip coating process as applied to a face of a disc, may also be applied to any other shape of substrate, such as, a flat rectangular shaped substrate of quartz.

5

PROCEDURE 4

The following sintering process may be carried out in standard laboratory furnaces of various sizes, capable of operating at temperatures from ambient up to at least 1100°C, and designed to maintain accurate and stable internal temperatures, particularly between 800°C and 1100°C, such as Lindberg models 51744 or 894-Blue M. The coated substrates as prepared by Procedure 3 are carefully transferred onto a standard ceramic plate (as is common practice in the Lindberg oven) - using, for example tweezers to avoid touching the coated surface. The ceramic plate is used as a carrier during the sintering process to facilitate easy loading and withdrawal of multiple substrates from the furnace. The furnace temperature is set to the temperature required to achieve the desired ratios of HA:α TCP. Utilizing a programmable furnace such as the Lindberg model 894-Blue M, the furnace may be programmed to hold the desired temperature, which will normally be selected from the range 920°C to 1100°C, for a maximum of one hour to ensure desired diffusion of the silicon entities through the developed gradient layers of hydroxyapatite and α-tricalcium phosphate. In the case of non-programmable furnaces, a separate timer may be used to warn the operator to turn the furnace off at the end of the required sintering time at selected temperature. The furnace is turned on and as it is heating to the required operating temperature, the ceramic plate carrying the substrates is quickly loaded at the point when the furnace approaches sintering temperatures, for example, 800°C. The furnace door is shut again immediately afterwards. The furnace is then allowed to reach the selected operating temperature for the required time and then turned off, either automatically or manually. The ceramic plate carrying the sintered

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substrates is removed at any time after the internal furnace temperature has cooled to an acceptable and safe touch-temperature of approximately 60°C. Individual substrates may then be stored or packaged for final use.

In accordance with this improved process, thin films of
5 hydroxyapatite/ α -tricalcium phosphate can be produced on a consistent basis having the desired composition where variability in the various processing parameters have been minimized to ensure such consistency.

Although preferred embodiments of the invention are described herein in detail, it will be understood by those skilled in the art that variations may be
10 made thereto without departing from the spirit of the invention..

THAT WHICH WE CLAIM:

1. A composition useful in providing a surface capable of supporting bone cell activity thereon, the composition comprising calcium phosphate entities developed by the conversion of hydroxyapatite at sintering temperatures into alpha tricalcium phosphate in the presence of

5

silicon entities.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant or Patentee: PUGH, Sydney M. et al Attorney Docket No.:
3477/104 Serial No. or Patent No.: 08/576,238
Filed or Issued: December 21, 1995
For: A Stabilized Thin Film of Calcium Phosphate Entities Particularly
Adapted for Bone Cell Culture

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act
on behalf of the concern identified below:

NAME OF CONCERN: Millenium Biologix Inc
ADDRESS OF CONCERN: 785 Midpark Drive, Suite 200, Kingston, Ontario K7M
7G3, Canada

I hereby declare that the above-identified small business concern
qualifies as a small business concern as defined in 37 CFR 121.3-18, and
reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under
Section 41(a) and (b) of Title 35, United States Code, in that the number
of employees of the concern, including those of its affiliates, does not
exceed 500 persons. For purposes of this statement, (1) the number of
employees of the business concern is the average over the previous fiscal
year of the concern of the persons employed on a full-time, part-time, or
temporary basis during each of the pay periods of the fiscal year, and (2)
concerns are affiliates of each other when either directly or indirectly
one concern controls or has the power to control the other or a third
party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to
and remain with the small business concern identified above with regard to
the invention, entitled A Stabilized Thin Film of Calcium Phosphate
Entities Particularly Adapted for Bone cell Culture by the inventors
Sydney M. Pugh; Timothy J.N. Smith and Michael Sayer described in:

- ☐ the specification filed herewith.
☒ Application No. 08/576,238 filed December 21, 1995
☐ Patent No. _____, issued _____.

If the rights held by the above-identified small business concern are not
exclusive, each individual, concern or organization having rights in the
invention is listed below* and no rights to the invention are held by any
person, other than the inventor, who would not qualify as an independent
inventor under 37 CFR 1.9(c) if that person made the invention, or by any
concern which would not qualify as a small business concern under 37 CFR
1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate
verified statements are required from each named person, concern or
organization having rights to the invention averring to their status as
small entities. (37 CFR 1.27)

NAME: _____

ADDRESS: _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME: _____

ADDRESS: _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Sydney M. Pugh

TITLE OF PERSON OTHER THAN OWNER: President

ADDRESS OF PERSON SIGNING: 785 Midpark Drive, Suite 200, Kingston,
Ontario K7M 7G3

SIGNATURE: _____

DATE: _____

ROOM
PR
2
1996

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

Attorney Docket No: 3477/104A

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **A STABILIZED THIN FILM OF CALCIUM PHOSPHATE ENTITIES PARTICULARLY ADAPTED FOR BONE CELL CULTURE**

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on December 21, 1995 as

Application Serial No. 08/576,238

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

English Language Declaration - Continued

Prior Foreign Application(s)

Priority Claimed

<u>(Number)</u>	<u>(Country)</u>	<u>(Day/Month/Year Filed)</u>	[] [] Yes No
<u>(Number)</u>	<u>(Country)</u>	<u>(Day/Month/Year Filed)</u>	[] [] Yes No
<u>(Number)</u>	<u>(Country)</u>	<u>(Day/Month/Year Filed)</u>	[] [] Yes No

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

60/003,157
(Application Number)

September 1, 1995
(Filing Date)

(Application Number)

(Filing Date)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>(Appln. Serial No.)</u>	<u>(Filing Date)</u>	<u>(Status)</u> (patented/pending/aban.)
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<u>(Appln. Serial No.)</u>	<u>(Filing Date)</u>	<u>(Status)</u> (patented/pending/aban.)
----------------------------	----------------------	---

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

English Language Declaration - Continued

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith (list name and registration number).

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Inventor's
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Date: _____

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Full name of second inventor: Timothy J.N. SMITH

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English Language Declaration - Continued

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Inventor's
Signature: _____

Date: 2/2/80

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Post Office Address: as above

Full name of fourth inventor:

Inventor's

Signature: _____

Date: _____

Residence:

Citizenship:

United States of America

Post Office Address:

EX-117 A



.....

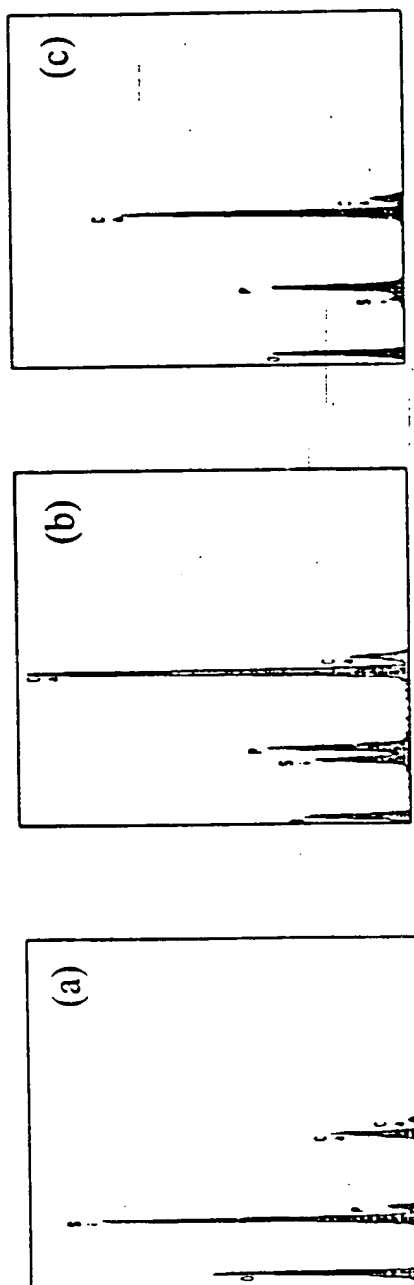


Figure 2. Energy Dispersive X-ray Spectroscopy of the Thin Film: (a) at and below the interface layer, (b) above the interface layer, (c) at the top of the film